W71-10639

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TOXIC ACTION OF WATER SOLUBLE POLLUTANTS ON FRESHWATER FISH

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INPUT TRANSACTION FORM

1 Accession Number: W71-10639

2 Subject Field & Group: 05C

5 Organization: Michigan State University, Department of Physiology, E. Lansing, ~~Mich. 48823~~

6 Title: Toxic action of water soluble pollutants on freshwater fish,

10 Author(s): ~~Dr.~~ Paul O. Fromm

11 Date: ~~10-1-70~~

12 Pages: ~~56~~

15 Contract Number:

16 Project Number: EPA-WQO Program 18050DST ~~Grant~~

21 Copy available from CFO Sup Doc ~~not~~ Paper Copy, $0.65 ; microfiche from NTIS as PB 201650, $0.95.

22 Citation: Water Pollution Control Research Series, December 1970, 56p, 11fig, 11tab, 30ref

23 Descriptors (Starred First)

Fish*, ~~stress~~*, chromium, insecticides*, ammonia*, sodium, ~~transport~~ ~~gill blood flow~~, nitrogen, *fish physiology, ~~nitrogen excretion~~ *Toxicity, *Trout, Dieldrin, Rotenone, *Rainbow trout,

25 Identifiers (Starred First)

~~Fish, stress~~, nitrogen excretion*, ~~fish physiology~~, *Goldfish, Malathion, MS-222, Sodium transport, Methoxychlor, Cell blood flow, Plasma (lab)

27 Abstract

Over a five year period experiments on rainbow trout indicated that exposure to chromium and to forced exercise caused a transient increase in plasma cortisol. Exposure to ammonia (a) caused a decrease in the rate of total nitrogen excretion and in ammonia excretion (b) caused some histopathological changes in trout gills but oxygen transport by hemoglobin was unaffected (c) caused a very slight increase in urea excretion by trout but a very significant rise in goldfish. Hyperexcitability observed in ammonia-exposed trout was not noticeable in the more resistant goldfish. Ammonia may kill fish by prevention of excretion of normal amounts of endogenous ammonia. Experiments with isolated-perfused gills of trout have shown among other things that (a) gill blood flow patterns are significantly affected by epinephrine (b) when perfused with Ringer solution there was a small but significant loss of sodium into the bath solution, whereas perfusion with sodium-poor Ringer solutions always resulted in a net uptake of sodium (c) perfusion fluid sodium and epinephrine appear to control sodium uptake by the gill (d) transfer of dieldrin into the vascular system occurred only when plasma protein, or more probably plasma lipoprotein was present in the perfusion fluid (e) short term exposure to dieldrin, rotenone, malathion and MS-222 reduced perfusion flow rate through isolated gills but exposure to 1 mg/L methoxychlor was without effect. Decrease in flow rate correlated well with lamellar perfusion.

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WR:102 (REV. OCT. 1968)
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TOXIC ACTION OF WATER SOLUBLE POLLUTANTS ON FRESHWATER FISH

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Water Quality Office

ENVIRONMENTAL PROTECTION AGENCY

Grant Number 18050 DST
December, 1970

For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402 - Price 65 cents

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ABSTRACT

In studies of the effect of stress on trout* we found that exposure to Cr^{+6} and to forced exercise caused a transient rise in plasma cortisol. Fasted fish treated with exogenous cortisol for 1 week had higher liver glycogen stores and excreted more waste nitrogen than controls, whereas data from fed animals were similar to control data. Ammonia appears to kill fish by preventing excretion of ammonia and not by interfering with gill respiratory exchange or inhibition of oxygen transport by hemoglobin. Under comparable conditions goldfish excrete much more urea than trout, and the excretion rates are much more responsive to ambient ammonia in goldfish. Hyperexcitability observed in ammonia-exposed trout was not noticeable in the more resistant goldfish. Greater histopathology was seen in trout gills than in goldfish gills exposed to the same or greater ammonia levels.

In studies using individual perfused gill arches of rainbow trout we found that: (a) the routes for blood flow are as described in previous studies using non-perfused gills; (b) significant changes in filamental and lamellar flow patterns appear to be controlled adrenergically, probably by vasoconstriction and vasodilation of sinus vessels and lamellar arterioles; (c) when perfused with Ringer solution there was a small but significant loss of sodium into the bath solution, whereas perfusion with sodium poor Ringer solutions always resulted in a net uptake of sodium; (d) perfusion fluid sodium and epinephrine appear to control sodium uptake by the gill; (e) uptake dependent on ATP energy produced aerobically was generally independent of the rate and pattern of fluid flow through the gill; (f) transfer of dieldrin into the vascular system occurred only when plasma protein, or more probably plasma lipoprotein was present in the perfusion fluid; (g) short-term exposure to dieldrin, rotenone, malathion and MS-222 reduced perfusion flow rate through isolated gills; exposure to 1 mg/L methoxychlor was without effect. Decrease in flow rate correlated well with increased lamellar perfusion.

This report was submitted in fulfillment of Grant No. 18050 DST under partial sponsorship of the Federal Water Quality Administration.

Key words: Fish, stress, chromium, insecticides, gill, sodium transport, gill blood flow, ammonia toxicity, nitrogen excretion.

*rainbow trout, Salmo gairdneri

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SECTION I

INTRODUCTION

The research conducted during the past five years represents an extension of earlier work (EF 00162) which was concentrated primarily on an investigation of the toxicity of chromium to fish. Although much of the work reported below has been published, few reprints are now available; hence the research is discussed giving sufficient details for understanding.

Except for the few experiments with goldfish, all fish used in the experiments discussed below were rainbow trout (Salmo gairdneri) supplied to us by the Michigan Department of Natural Resources hatcheries at Harrietta or Grayling, Michigan. All arrangements for supplying fish were made with Dr. L. N. Allison, Fish Pathologist, who also offered advice on problems relating to husbandry and diseases of hatchery fish. All fish were held in the laboratory in 300 liter tanks lined with fiberglass for at least one week prior to use. They were fed commercial trout pellets during the phase of acclimation to laboratory conditions. Treatment and feeding of fish varied with each experiment and are indicated below.

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SECTION II

RESPONSE OF THE INTERRENAL GLAND OF RAINBOW TROUT TO STRESS

A series of experiments were undertaken to determine whether chronic exposure to low levels of noxious stimuli will cause an increase in plasma levels of glucocorticoids in rainbow trout. We also investigated some of the metabolic effects of artificially elevated levels of glucocorticoids. At the outset of these investigations it was considered that the cortisol assay procedure of Guillemin et al. (1959) was the method of choice. Since that time others have shown that abbreviated procedures such as the one used can lead to erroneous results. It is known that the principal steroid elaborated by the interrenal of rainbow trout is cortisol, but there are fluorogens other than cortisol also present such as 20 β dihydrocortisone. Most of the plasma samples had been processed before we realized that in order to state categorically that the changes in fluorescence of plasma that we measured were due to cortisol alone, we should have isolated cortisol (chromatography) and then measured it fluorometrically. Our strongest argument for stating that the changes we observed were due to changes in plasma cortisol comes from the fact that when cortisol pellets were implanted in fish we were able to determine a significant elevation in the fluorescence of the plasma extracts.

Chromium stress: Two groups of fish were held in water containing 0.02 and 0.20 mg Cr/L; controls were held in aged tap water. At these levels radiochromium was found to accumulate steadily in rainbow trout throughout a 28-day experimental period (Fromm and Stokes, 1962). Fish exposed to 0.2 mg Cr/L for 1 week had plasma cortisol levels nearly twice those of controls (54.3 vs. 30.5 µgm/100 ml). Those fish exposed to the lower concentration also had significantly elevated plasma cortisol. After exposures of 2 and 3 weeks the Cr-treated fish had plasma levels essentially similar to those of controls. Fish exposed to 20 mg Cr/L for 3 days showed plasma cortisol levels of 56.8 µg/100 ml compared to 37.8 for controls.

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Fish exposed for 6 and 7 days showed no increase in blood levels over controls; and those exposed 10 days had somewhat elevated plasma cortisol, but they were not statistically higher than values for controls.

Effect of exercise: Fish were given two daily half-hour periods of forced exercise for one week and two weeks in separate experiments. The speed of rotation of the circular tank used was regulated so that the fish were swimming as rapidly as they were willing to do for the half-hour period.

Table I

Plasma cortisol levels in fish exposed to forced exercise for 2 or 4 hours. Blood samples taken immediately after cessation of exercise.

Duration (hours)	Number of data	Plasma cortisol (μg/100 ml)*
Control	5	54.4 ± 3.5
2	5	61.0 ± 2.3 ($p > 0.05$)
4	5	64.2 ± 4.3 ($p < 0.05$)

* mean ± S.E.

When the blood samples were taken 24 hours after cessation of exercise the exercised fish had cortisol levels slightly lower than those for controls. In a second series of experiments fish were forced to swim for 2 and 4 hours and blood samples were taken immediately after capture. Plasma cortisol levels in fish exercised for 2 hours were somewhat elevated above those for control fish, and the elevation after 4 hours of exercise was statistically significant.

Metabolic effects of exogenous cortisol: To study the effects of elevated serum cortisol, exogenous cortisol in cholesterol pellets was administered to fish by intraperitoneal implantation. Pellets of cholesterol only were implanted in controls. Some characteristic effects of elevated levels of adrenal cortex activity in mammals include

an increase in liver glycogen, an elevation in plasma glucose, and an increase in the utilization of amino acids for energy metabolism and gluconeogenesis, with a resulting increase in nitrogen excretion. These metabolic parameters were measured in fish that received intraperitonally implanted pellets containing cortisol.

Table II

Effects of 1-week exposure to exogenous cortisol on Nitrogen Excretion, Liver Glycogen and Plasma Glucose of fasted rainbow trout.

Parameter	Control (6)*	Experimental (8)*
Plasma cortisol (μg/100 ml)	48.7 ± 4.5	125.7 ± 16.0
Liver glycogen (g/100 g tissue)	0.59 ± 0.16	1.17 ± 0.12
Nitrogen excretion (mg N/kg/day)	219 ± 29	306 ± 61
Plasma glucose (mg/100 ml)	86.3 ± 11.5	74.1 ± 11.0

* all data: Mean ± S.E.; number of data in parentheses.

In fasted experimental fish the glycogen content of the liver and values for nitrogen excretion were higher than those for controls. Plasma glucose in treated fish were similar to values for controls. A similar experiment was conducted with fish that were fed at a rate of 2% of body weight per day during the period between implanting of pellets and sampling. Neither liver glycogen levels nor nitrogen excretion was elevated in the cortisol-treated fish which had been fed.

Table III

Effect of 1-week exposure to exogenous cortisol on Nitrogen Excretion and Liver Glycogen of fed rainbow trout.

Parameter	Control (7)*	Experimental (6)*
Liver glycogen (g/100 g tissue)	3.63 ± 0.69	3.55 ± 0.56
Nitrogen excretion (mg N/kg/day)	348 ± 77	326 ± 12

* all data: Mean ± S.E.; number of data in parentheses.

Discussion and summary: Exposure to chromium resulted in a transient elevation in the concentration of fluorogenic materials in the plasma of rainbow trout. The response of fish to 20 mg Cr/L, a concentration approaching the 48-hour median tolerance limit for the species, caused a response that was of a much lower magnitude than that observed in spawning Pacific salmon, which, according to Robertson and co-workers, causes detrimental degenerative secondary responses. Results somewhat similar to ours were obtained by McKim (1966), who measured output of urinary 17 hydroxycorticosteroids (17-OHCS) metabolites in rainbow trout exposed to a continuous stress of sublethal environmental levels of the detergent, alkyl benezene sulfonate (ABS). Excretion rates of 17-OHCS of experimental fish increased about twofold in the first 24 hours of exposure but, in all cases, decreased to only slightly above (at 7 mg ABS/L) or equal to (at 3 and 5 mg ABS/L) the rates of control fish at the end of 7 days. There is reasonable doubt as to whether the untreated controls for the chromium study represented unstressed fish, since there was some elevation of cortisol levels during the 3-week experimental period and we have no plausible explanation of the increase observed. Variations of interrenal activity may have been influenced by the social interaction of fishes, although no observations of this interaction were made in the present study. Erickson (1967) found that green sunfish in small aquaria established a social hierarchy and that fish of lower social rank, presumably the more stressed individuals, had significantly greater volumes of interrenal tissue. He found that the quantity of interrenal tissue present was negatively

correlated with the number of aggressive actions that the fish initiated. During the course of our study we noted considerable variability in the values for plasma cortisol in controls that may reflect a possible seasonal variation in the activity of the interrenal tissue of trout and/or variations in the rate of clearance. There is evidence that the responsiveness to ACTH of the adrenal cortex of rats and humans is decreased by high circulating corticoid levels in the blood. Hane et al. (1966) injected mammalian ACTH and caused a fourfold increase in plasma 17-OHCS in nonspawning Pacific salmon captured at sea and similar results in sexually undeveloped fish captured at the beginning of their spawning migration. Responsiveness to ACTH decreased as the fish approached sexual maturity, coincident with increased plasma 17-OHCS, and little response was seen in spawned fish whose plasma 17-OHCS levels were greatly elevated. Any seasonal variation in plasma cortisol levels of untreated fish is of particular significance to anyone wishing to use this parameter as an indicator of stress. Direct comparisons of values obtained at different times of the year would be valid only if completely adequate control groups could be used. Short-term stress has consistently resulted in elevated circulating glucocorticoid levels in fish in our experiments and those of others (Hatey, 1958; McKim, 1966; Fagerlund, 1967). There is evidence from McKim's ABS data and from our chromium experiments that the magnitude of the response can be expected to vary with the magnitude of the noxious stress. This response might be useful for evluating stress in short-term studies. The apparent important role of elevated circulating corticosteroids, whether the result of increased activity of the interrenal gland or impaired clearance, in bringing about the degenerative changes observed in spawning salmon has been given strong support by the successful reproduction of most of these changes by the administration of exogenous cortisol to immature rainbow trout (Robertson et al., 1963). The high corticosteroid levels found in migrating Pacific salmon may be an unusual phenomenon associated with the simultaneous demands of long-sustained exertion of migration and of maturation of a large mass of gonadal tissue and gametes. Our results, as well as those of McKim (1966) and Fagerlund (1967), support the idea that the response of the interrenal gland of fish to chronic sublethal stress does not play a major role in the genesis of adverse reactions that may be caused by these stressors. As noted above, analysis of the response of this tissue during short-term studies of stress may be quite valuable.

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SECTION III

EFFECT OF AMMONIA ON TROUT AND GOLDFISH

Effect of ammonia on trout: Deamination of amino acids by the liver, metabolic activity of nerve and muscle tissue, as well as activity of enzymes contained in the flora of the gut on substrates derived from the diet and the blood, all lead to the production of ammonia. Ammonia is quite toxic to most organisms, and it must be either continually eliminated or converted to less toxic compounds to prevent any buildup to harmful concentrations within the body. When the pH of an aqueous solution of ammonia is increased, the amount of non-ionized ammonia is increased. The free base (NH_3) is able to diffuse across cell membranes easily because of its lipid solubility and lack of charge, whereas the ammonium ion penetrates membranes less readily because it is hydrated, charged and has a low lipid solubility. This being the case, one would expect that the pH of an ammonia solution would have a great effect on the toxicity of the solution. The toxicological actions of ammonia on fish are not completely known. Burrows (1964) has observed extensive proliferation and consolidation of gill lamellae of salmonids exposed to ammonia and similar observations have been reported by Reichenbach-Klinke (1967). These affected fish were quite susceptible to gill disease, and Burrows regards the ammonia irritation as a precursor to the disease. The consolidation of lamellae reduces the surface area of the gills and thereby reduces the ability of the fish to liberate CO_2 and to absorb oxygen. Brockway (1950) has correlated an increase in ambient ammonia with a reduction in oxygen level of the blood and suggested that ammonia affects the oxygen transport ability of fish blood. If ammonia is to act internally, it appears probably that blood levels of ammonia should increase in fish concurrent with an increase in ambient ammonia. Any change in blood ammonia under these conditions could be due to either inhibition of excretion or inward diffusion of ammonia. The aims of the experiments described below were to investigate changes that occur in blood ammonia levels and in ammonia excretory rates when fish are exposed to increased concentrations of ambient ammonia. Data for daily excretion of total nitrogen were obtained to see if any change in excretion rate or form of nitrogenous waste occurred in fish exposed

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to different concentrations of ambient ammonia. We also investigated the effect of ammonia on the oxygen carrying ability of hemoglobin.

When rainbow trout were exposed to increased ambient concentrations of total ammonia, the levels of total ammonia in the blood increased. Slight differences in the pH of the water samples was noted which altered the proportion of NH_3 and NH_4^+ in samples of equal total ammonia concentration. When blood ammonia, total and unionized, was plotted versus ambient ammonia (total and unionized, respectively), both graphs indicated a direct linear correlation between blood ammonia and water ammonia. Equations for the slopes determined by the method of least squares are given in Figures 1 and 2. In all cases the concentration of total and non-ionic ammonia in the blood was higher than their respective concentrations in the water from which the fish were taken.

No measure of the external action of ammonia on trout was made in our experiments, but it was certainly obvious that fish placed in the high concentrations of ammonia were hyperexcitable. Wuhrmann & Woker (1948) and McCay & Vars (1950) reported similar observations. We have no information on the production of ammonia by neural tissue or the effects of ammonia on the excitability and/or metabolism of neural tissue of fish. In mammals there are indications that the formation of ammonia by nerve tissue is due to reactions involving proteins and nucleoproteins; however, details concerning the nature of its precursors and the mechanism of its liberation are lacking. The main stream of ammonia disposal is the combination of one molecule of ammonia with α-ketoglutarate to form L-glutamate. Then a second molecule of ammonia combines with glutamate, with the utilization of an ATP, to form glutamine, a compound which traverses the blood-brain barrier more easily than glutamate. An auxillary mechanism utilizing transamination exists. L-glutamate, pyruvic and oxalacetic acids are involved, with the resultant formation of alanine and asparate. When the ammonia concentration is increased, it appears to stimulate glycolysis in neural tissue. The end-result of excessive ammonia production is an accumulation of pyruvate and lactate brought about by stimulation of the glycolytic pathway and concurrent suppression of citric acid cycle activity due to a diversion of α-ketoglutarate and oxalacetate into increased amino acid production. Although the ammonia binding mechanisms may give rise to many of the effects associated with increased production of ammonia, Weil-Malherbe (1962) concludes that they are necessary in order to prevent accumulation of free ammonia, which would be even more detrimental to the organism.

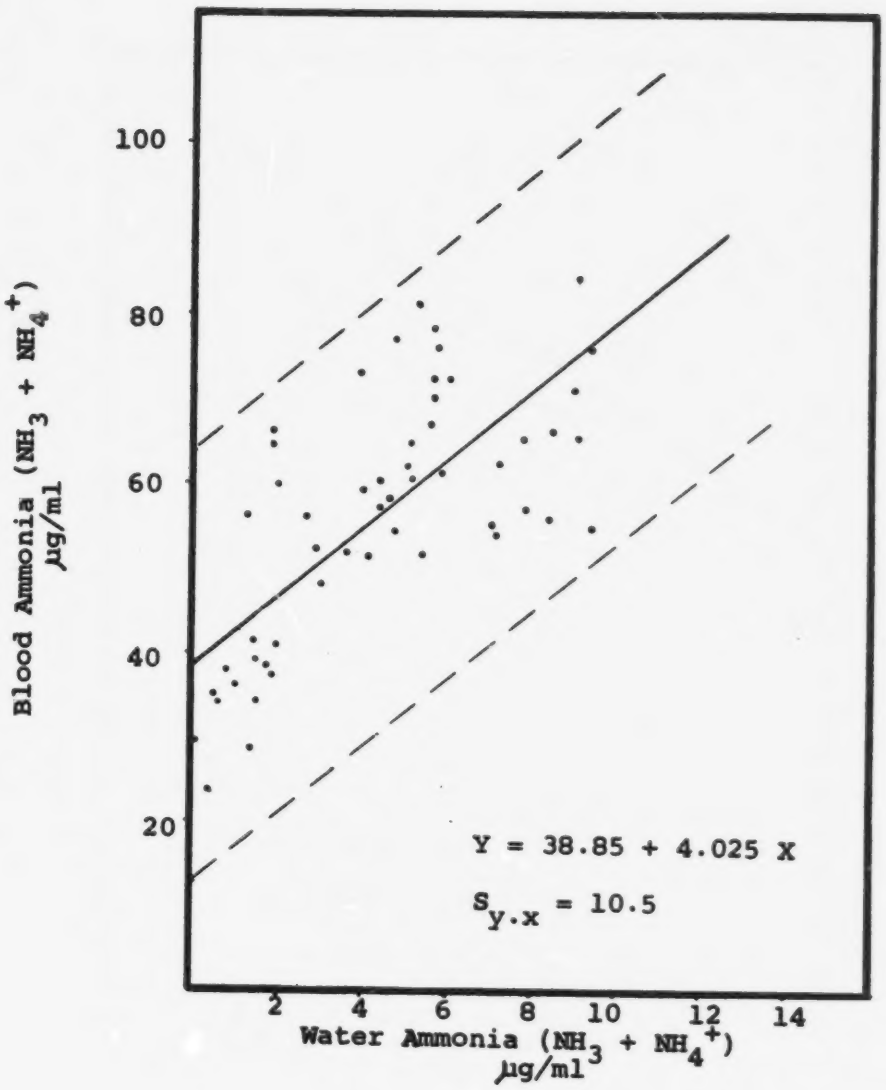

Figure 1. Total ammonia concentration in the blood as a function of total ammonia concentration in the water. The solid line was drawn using the method of least-squares. Ninety-five per cent confidence limits for points on this line are indicated by the dotted lines.

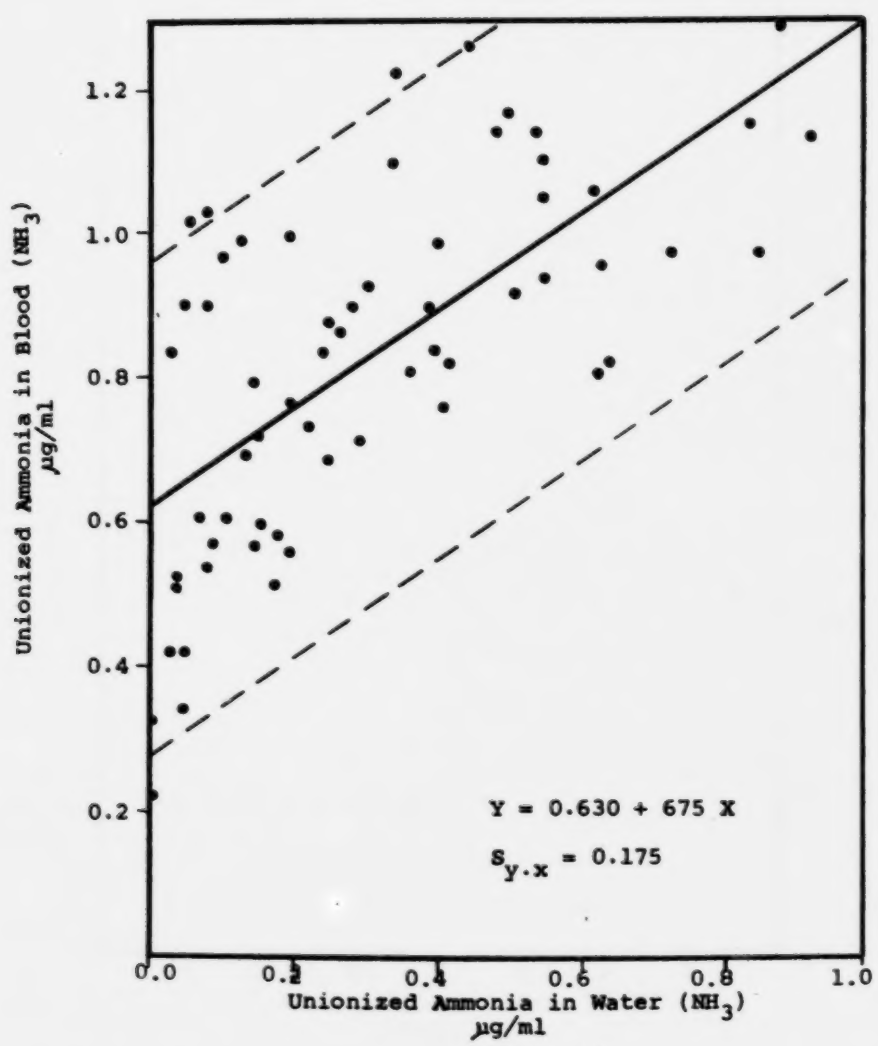

Figure 2. Non-ionic ammonia concentration in the blood as a function of non-ionic ammonia concentration in the water. The solid line was drawn using the method of least-squares. Ninety-five per cent confidence limits for points on this line are indicated by the dotted lines.

Ammonia in the water appears to be toxic to trout by suppressing the liberation of ammonia at the gill surface. Since the blood ammonia level always exceeded that in the environment from which the fish were taken, the source of the blood ammonia must have been endogenous. Thus the rate of metabolic production and/or release of ammonia into the blood exceeded the combined rates of excretion and detoxification in these fish. Evidence for detoxification of ammonia was obtained by experiments in which the rate of total waste nitrogen and ammonia nitrogen excretion was determined for fish exposed to various concentrations of ambient ammonia. As the water ammonia level increased, total nitrogen excretion decreased. Ammonia excretion decreased also and the decrease in ammonia excretion could account for about 48% of the decrease in total nitrogen excretion up to an ambient concentration of 5 μg/ml. At an ambient concentration of 8 μg/ml the amount of ammonia excreted was greatly reduced but excretion of total nitrogen remained quite high, indicating that the reduction in ammonia excretion was to some extent compensated for by increased excretion of some other nitrogenous compound.

Brockway (1950) reported that when the ammonia in water increased to about 1 mg/L the oxygen content of trout blood decreased to approximately one-seventh of its normal value, and the carbon dioxide content increased about 15%. One might argue that the external action of ammonia on the gill epithelium could affect respiratory exchange and give rise to the alterations noted in the blood gases. In experiments (unpublished) we found that rainbow trout exposed to 1.6-4.3 mg NH_3/L for 24 hours had rates of oxygen consumption which ranged from 53 to 106 per cent above the normal resting level. In these fish the exchange of respiratory gases was not impaired by ammonia, although the rates of oxygen usage and carbon dioxide production could have given rise to alterations in the blood gas content similar to those reported by Brockway.

To test the effect of ammonia on the ability of hemoglobin to combine with oxygen in vitro, the following procedures were followed. Whole blood, obtained from untreated fish, was centrifuged and the plasma removed. To a volume of cells an equal volume of ammonia-free Ringer solution was added, and to aliquots of this mixture equal volumes of Ringer solution which contained known quantities of ammonia were added. These RBC-Ringer mixtures, which had hematocrit values of about 25 per cent, were then aerated by bubbling air through them for a minimum of 5 minutes immediately prior to the analyses given below. During all of the procedures the temperature of the samples was maintained at 12°C, and the pH of the final mixtures

Table IV

Excretory nitrogen components from rainbow trout subjected to various levels of ambient ammonia. Values for nitrogen excretion are as μg N/gm of fish/day. The per cent N accounted for is: excreted ammonia N + urea N + protein N × 100/Total N excreted.

Mean ambient ammonia (μg/ml)	n	Ammonia N excretion	Urea N excretion	Protein N excretion	Total N excretion	Per cent N accounted for
2.39 ± 0.09*	19	86 ± 7.8	39 ± 7.6	35 ± 4.9	161 ± 15.9	99
4.58 ± 0.17	12	63 ± 7.2	28 ± 5.8	44 ± 9.0	151 ± 19.9	89
6.05 ± 0.13	10	35 ± 4.5	30 ± 4.9	24 ± 4.3	91 ± 11.0	97
8.30 ± 0.21	17	24 ± 6.9	30 ± 3.4	38 ± 4.2	103 ± 10.1	89

* Standard error of mean.

was about 7.4. After aeration, aliquots were taken for determination of oxygen content using the Natelson Microgasometer and hemoglobin content by the acid hematin method (B & L Spectronic 20 Clinical Technique Manual). It was found that ammonia in concentrations up to 10 µg/ml had no significant effect on the ability of trout hemoglobin to combine with oxygen in vitro.

In general summary our results indicate that ammonia in water (1 ppm as NH_3) is toxic to trout because of its effect of preventing the excretion of normal amounts of ammonia. The toxic action most probably is at the cellular level, and the nervous system appears to be affected earliest. Ammonia at the levels studied does not appear to kill fish by preventing exchange of the respiratory gases at the gill surface or by inhibiting transport of oxygen in combination with hemoglobin.

In another series of experiments teleosts which inhabit distinctly different environments were studied to determine if any species variability to ammonia toxicity exists. Rainbow trout, which require relatively clean water, and goldfish (Carassius carassius), which have a tolerance for stagnant water, were used.

Trout subjected to increased ambient ammonia with no previous acclimation period showed a decrease in total nitrogen excreted (Table IV) and a concomitant decrease in waste nitrogen excreted as ammonia. Except for a slight increase in urea excretion at low ambient ammonia, urea and protein nitrogen excretion rates showed no change as ambient ammonia increased. Ninety-four per cent of the total nitrogen excreted by trout was as ammonia, urea and protein nitrogen. Two groups of trout were then acclimated to high (5 µg/ml) and low (0.5 µg/ml) ambient ammonia and then subjected to about 3 µg/ml while their urea excretion rate for 24 hours was determined. Those exposed to high ammonia actually excreted slightly less urea than those exposed to low ammonia, which was just the opposite of what was expected.

In studies with goldfish, determinations of urea excretion rates were made, but excretion of total waste nitrogen was not investigated. Fish were acclimated to low and high ambient ammonia, and then the rate of urea excretion was determined at the concentrations as indicated in Table V. It is apparent from the data that pre-conditioning or acclimation to high ammonia has little or no effect on urea excretion. Urea excretion rate is dependent on the ambient ammonia levels during the collection period and is independent of any acclimation

concentration or duration of acclimation. The goldfish appear to respond to a change in ambient ammonia almost instantaneously.

Table V

Urea excretion during 24 hours at various levels of ambient ammonia by goldfish which had been acclimated to either low (0.5 µg/ml) or high (5.0 to 25.0 µg/ml) ambient ammonia.

Days fish acclimated	Acclimation concentration µg ammonia/ml	n	Ambient concentration during experiment µg ammonia/ml	Urea excretion µg N/gm of fish/day
26	0.5	7	2.37 ± 0.23*	134 ± 31
46	0.5	3	0.75 ± 0.03	52 ± 7
56	0.5	7	0.08 ± 0.01	27 ± 3
26	5.0	11	2.22 ± 0.14	128 ± 21
20	25.0	4	0.68 ± 0.11	22 ± 10
30	25.0	9	0.10 ± 0.01	28 ± 2

* Standard error of mean.

Trout placed in water containing more than 3 µg ammonia/ml became hyperexcitable. Any disturbance of the tank or movements above the tank visible to the fish resulted in disoriented escape attempts which sent the fish crashing into the sides of the tank. If these fish were then placed in water containing no ammonia, they appeared to return to normal, i.e., were no longer hyperexcitable. The highest ammonia concentration (about 8 µg/ml) to which trout were exposed caused about 50 per cent mortality within 24 hours. There was a decrease in mortality with the corresponding decrease of ambient ammonia concentrations during the 24 hour period. The onset of

death was characterized by violent thrashing movements which were functionless as propulsive swimming movements. Trout used in the high ammonia acclimation experiments were also hyperexcitable initially; however, after about two days they appeared to calm down, and after the third day they showed no signs of elevated excitability. Conversely, goldfish did not appear to be bothered at all by ammonia as high as 25 µg/ml, some eight times greater than the concentration which affected the trout. When the goldfish were placed in 40 µg ammonia/ml, about 10 per cent died in 24 hours. The onset of death was characterized by a gradual cessation of swimming movements, during which time the fish slowly settled to the bottom of the aquarium. After one or two hours nearly all of the dying fish curled laterally in the form of a "U" and opercular movements dropped considerably. If left in the ammonia solution, death soon followed. Three fish near death were removed from the ammonia water and placed in ammonia-free water; two of the fish lived for several days before dying, and the third completely recovered. Although severe, the effects of ammonia on fish apparently are, to some extent, reversible.

The results of the experiments with trout and goldfish can be briefly summarized as follows:

When rainbow trout were subjected to increasing ambient ammonia concentrations at 13°C, the total nitrogen excreted decreased, which is reflective of a decreased excretion rate of ammonia. Except for an initial increase of urea excretion at very low ambient ammonia levels, urea- and protein-N excretion rates remained constant at the other levels tested. An average of 94 per cent of the total nitrogen excreted by trout consists of ammonia, urea and protein nitrogen. Rainbow trout acclimated to elevated ammonia levels showed no increase in urea excretion over that of controls acclimated to low ammonia.

When goldfish were exposed to increased ammonia levels at 20-23°C, the rate of urea excretion increased. Acclimation of goldfish to different elevated ammonia levels for variable periods of time show that the change in rate of urea excretion was dependent solely on the ambient ammonia level during the experimental period and was unaffected by prior treatment. The ability of goldfish to change their urea excretion rate concomitant with a change in ambient ammonia appears to be either instantaneous or with a time course so short that any lag time is insignificant in 24 hours.

Four photomicrographs of gill lamellae from rainbow trout appear on the following pages. The sections were cut at 8 microns and stained with hematoxylin and eosin. Descriptions of these figures are as follows:

Figure 3. Photomicrograph of gill lamellae from rainbow trout exposed to low ambient ammonia (< 0.5 µg/ml) for 8 weeks. Gills exposed to this very low level of ammonia have long slender lamellae which exhibit no significant pathology. (× 133)

Figure 4. Photomicrograph of gill lamellae from rainbow trout exposed to 5 µg ammonia/ml for 8 weeks. The lamellae are shorter and thicker than those seen in Figure 3, and they have bulbous ends. Some consolidation of lamellae was also noted in fish exposed to high ammonia, 5 µg/ml. (× 133)

Figure 3

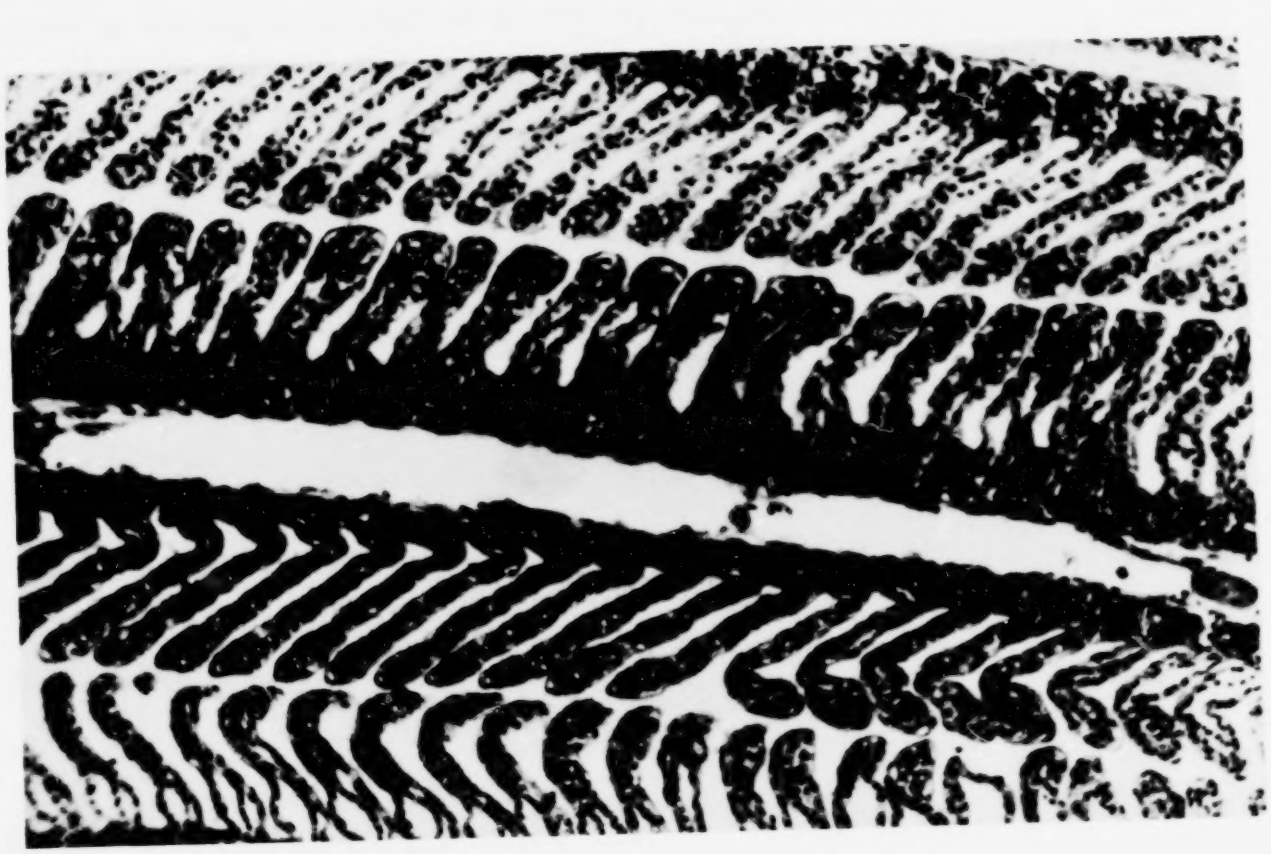

Figure 4

Figures 5 and 6. Higher magnification (× 532) photomicrographs of lamellae from trout exposed to 5 µg ammonia/ml for 8 weeks. Two types of pathology can be recognized. Many filaments show a rather limited hyperplasia (Figure 5) which is accompanied by the appearance of cells containing large vacuoles whose contents stain positive for protein. Other lamellae (Figure 6) show a definite hyperplasia of the epithelial layer, as is evident by an increase in the number of cell nuclei.

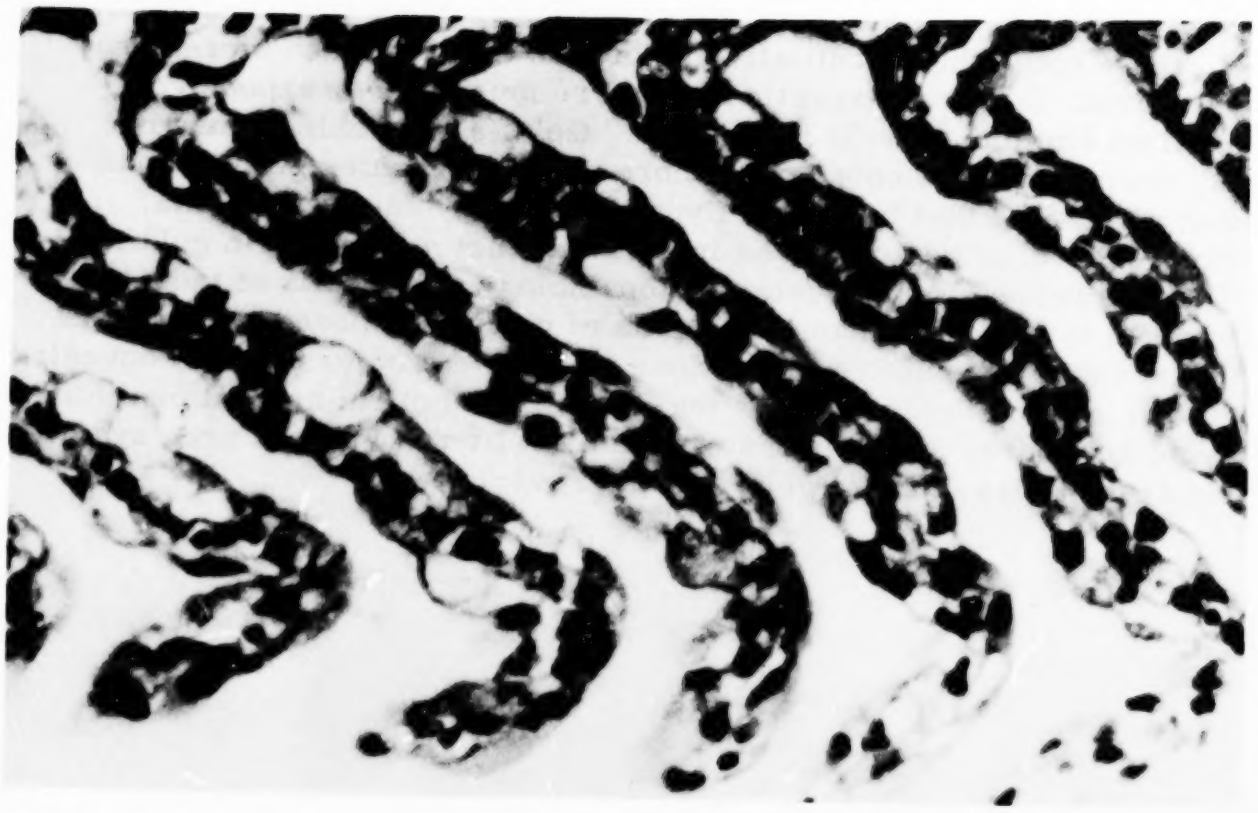

Figure 5

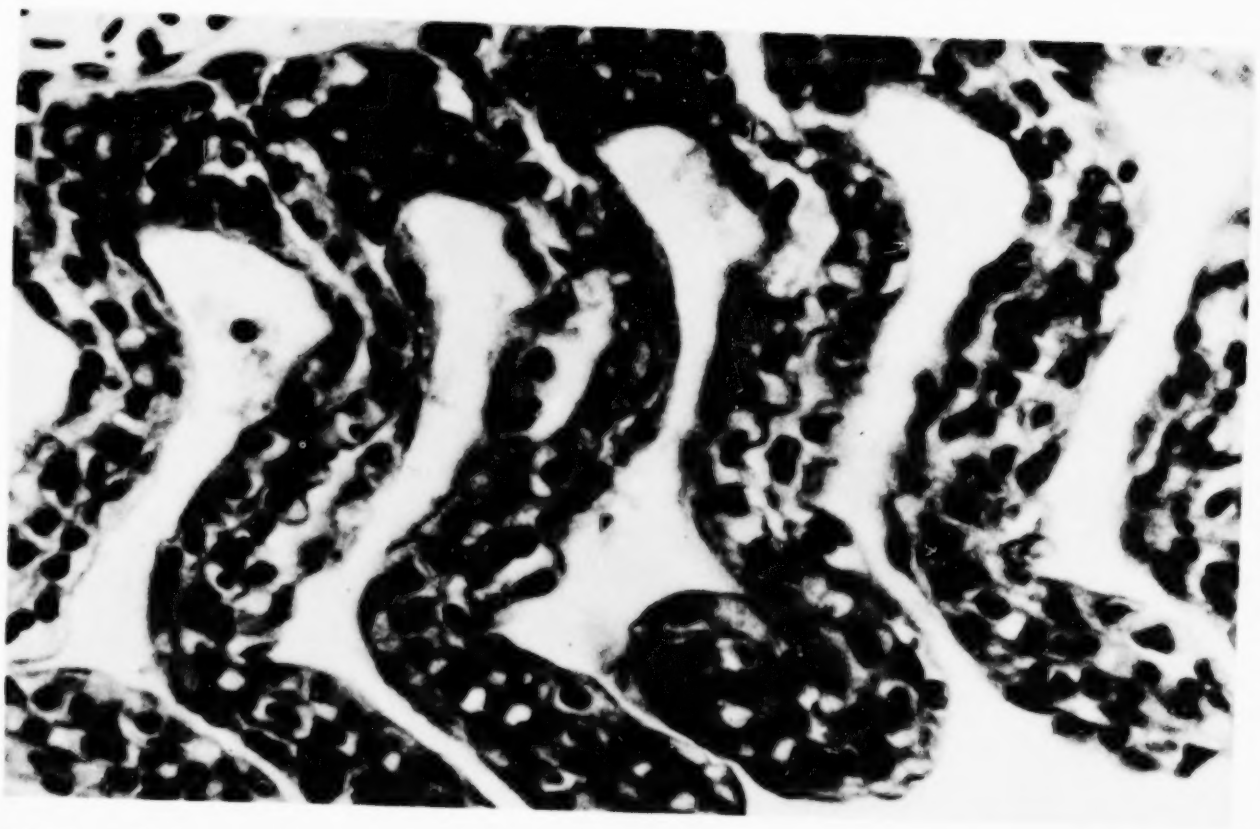

Figure 6

With regard to species variability between rainbow trout and goldfish, it can be noted that quantitatively goldfish excrete much more urea than trout, and urea excretion rates are much more responsive to ambient ammonia levels in goldfish. Goldfish are able to survive (0% mortality) ammonia levels more than three times greater than that which is lethal to trout. Hyperexcitability due to ammonia exposure which was observed in trout was not noticeable in goldfish. There appeared to be greater histopathology of the gills of trout exposed to ammonia than to the gills of goldfish exposed to the same or greater ammonia concentrations. Enzyme studies by other investigators support the hypothesis that purine catabolism and not ornithine cycle activity is probably the main metabolic pathway for urea synthesis at the rates found in goldfish and trout.

SECTION IV

STUDIES USING

ISOLATED-PERFUSED RAINBOW TROUT GILLS

The gill surfaces used in respiratory exchange by most aquatic animals such as fish are maximally exposed to water borne or water soluble pollutants. It has been recognized for years that heavy metal ions have an effect on gills, but much of the research accomplished to date has been biased towards the determination of lethal concentrations for a particular pollutant. Of all organs present in fish, we figure that the gills receive the greatest initial insult when these animals are placed in polluted waters. A toxic agent may act externally on the gills causing, for example, erosion or precipitation of gill mucus; or it may act internally, bringing about its deleterious effect by interfering with metabolic cycles. We originally planned to perfuse the whole branchial apparatus of trout, measure selected physiological parameters and then test the effect of certain chemicals on this preparation. We finally gravitated to the use of a single gill arch in order to enhance our chances of doing more quantitative measurements. Early studies indicated that the pattern of blood flow through isolated gills was a significant variable with which we had to contend. Experiments using the isolated gill technique have produced some interesting results and the technique, slightly modified, is currently being used in new experiments in our laboratory.

The initial aim of this perfusion study was to obtain data for "normal" or control level of sodium transport by gill epithelial cells. The gills were bathed with a 1 per cent Ringer solution and perfused with 100 per cent Ringer solution. Under these conditions no measurable uptake of sodium occurred. We then lowered the internal sodium concentration by a partial substitution of sodium chloride with choline chloride. It had previously been suggested that acetylcholine caused a shift in the blood flow pattern through the filaments of teleosts, thus it was necessary to investigate the flow patterns in trout gills in order to ascertain whether or not choline chloride affected flow. To do this we perfused gills with Ringer solutions containing India ink along

with the material to be tested. Histological preparations of these gills were examined.

Gill blood flow: Rather extensive studies were made of the patterns of blood flow through filaments and lamellae of isolated-perfused rainbow trout gills. To do these experiments we used the constant-pressure perfusion apparatus shown diagrammatically in Figure 7. Prior to cannulation, the dorsal and ventral ends of the gill were trimmed of excess bone and tissues, exposing the efferent and afferent branchial arteries. The valve on the perfusion apparatus was opened and a steady, rapid flow of fluid was established. The afferent cannula was then inserted into the afferent branchial artery and tied in place with No. 30 cotton thread. The ligature was tied around the arch rather than directly around the artery, thus preventing loss of fluid due to leakage at the cut end of the arch. The efferent artery was cannulated in the same manner, and the gill was immersed in 50 ml of 1 per cent Ringer solution. The placement of the ligatures was such that approximately 15 per cent of the filaments were outside of each ligature, and thus only about 70 per cent of the filaments on a given arch were perfused. Gills were first perfused with Ringer solution containing the test substance (control, 10^{-5}M epinephrine, 10^{-5} acetylcholine). These perfusions were carried out with the free end of the efferent cannula at approximately the same height as the gill and were continued until no more blood could be seen entering the collecting tube. The volume of perfusate collected during this period was never less than 100 µL. At the end of the initial perfusion, the free end of the efferent cannula was raised to a height of 20 cm above the gill and was inserted into another 100-µL pipette. The valve on the perfusion apparatus was then turned and India ink, which contained the test substance in the concentrations as noted above, was allowed to flow through the gill until at least 100 µL of ink solution had been collected from the efferent cannula. The ink used was Pelikan Biological India Ink (John Henschel Co., New York). According to Peterson et al. (1965), this ink contains 10 per cent carbon with a particle size of 0.02-0.03 µ, 4.3 per cent fish glue, 1.0 per cent phenol, and none of the shellac or ammonia normally found in other ink preparations.

Additional ligatures which had been placed around the ends of gill arches were tightened as soon as the cannulas were removed at the end of the perfusion period, thus minimizing loss of ink from the branchial vasculature during subsequent processing. Gills were placed in Dietrich's fixative (10 parts formalin, 20 parts 95% ethanol, 2 parts glacial acetic acid, and 59 parts water) for 12 hours, then

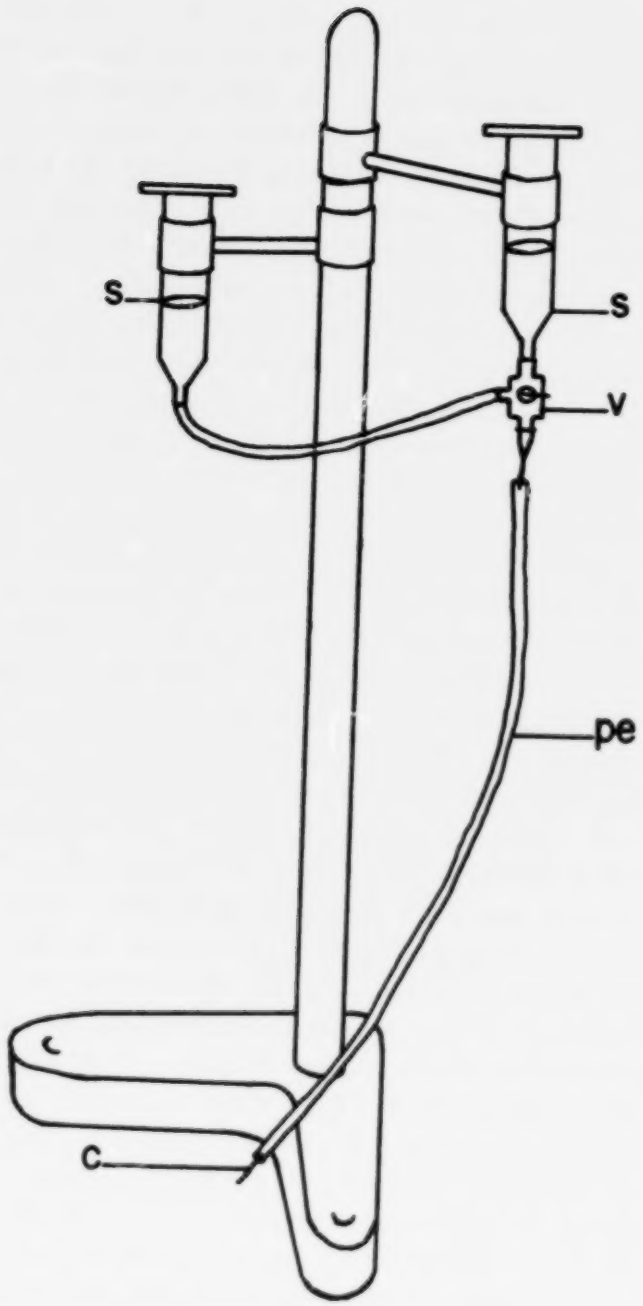

Figure 7. Constant pressure perfusion apparatus: s, syringe; v, valve; pe, polyethylene tubing; c, cannula.

dehydrated, infiltrated with and embedded in Paraplast (A. H. Thomas, Co., Philadelphia) using standard procedures. Sections of gills 8 μ thick were mounted on glass microscope slides and stained using standard procedures of Masson's trichrom stain. Only gills from which there was no visible leakage of India ink during perfusion were prepared for histological examination. In fish, blood flows from the ventral aorta into afferent branchial arteries, thence to afferent filamental arteries, through lamellae, collects in efferent filamental arteries, then flows into efferent branchial arteries and into the dorsal aorta. Filamental and lamellar circulation were studied in detail, for it is in these parts where exchange of materials between the blood and the environmental water takes place. The India ink distribution in acetylcholine-treated gills was essentially identical to that seen in untreated controls. In both cases the ink was concentrated in the filamental sinus and collateral vessels, with very little ink appearing in the lamellar lacunae or marginal channel (see Figures 8 and 9). In the epinephrine-treated gills the ink was concentrated in the lamellae, with little or no ink in the filamental sinus and collateral vessels. In gills perfused with India ink made up in "choline Ringer solution," the distribution of ink resembled much more closely that seen in acetylcholine-treated and control gills than it did that in epinephrine-treated gills. The distribution of ink in gills perfused with "choline Ringer solution" containing 10^{-3}M atropine, however, was more similar to the pattern seen in epinephrine-treated gills than to that seen in acetylcholine-treated and control gills. Histological cross-sections of the afferent-lamellar and efferent-lamellar arterioles revealed that both of these vessels contain muscular elements within their walls. The presence of contractile elements in the walls of the efferent-sinus vessels has not been clearly demonstrated histologically, but neither the dimension of the vessels themselves nor the thickness of the vessel walls precludes such a possibility. It was also observed that the rate of fluid flow through the gill was strongly affected by the nature of the perfusing fluid. The addition of epinephrine to the fluid caused a 177 per cent increase in flow rate and perfusion with "choline Ringer solution" resulted in a 66 per cent reduction in flow rate. Addition of atropine to the "choline Ringer solution" resulted in a flow rate which was not significantly different from the control rate. In their studies of the pattern of blood flow through the gill, Steen & Kruysse (1964) placed freshly excised gill filaments into physiological salt solutions on a microscope slide. They then placed a cover-slip over the filament and observed the patterns of fluid flow when pressure was applied to the cover-slip. Using this method, they found that in untreated gills blood flowed, often simultaneously, between the afferent and the efferent filamental arteries by way of the (a) lamellae,

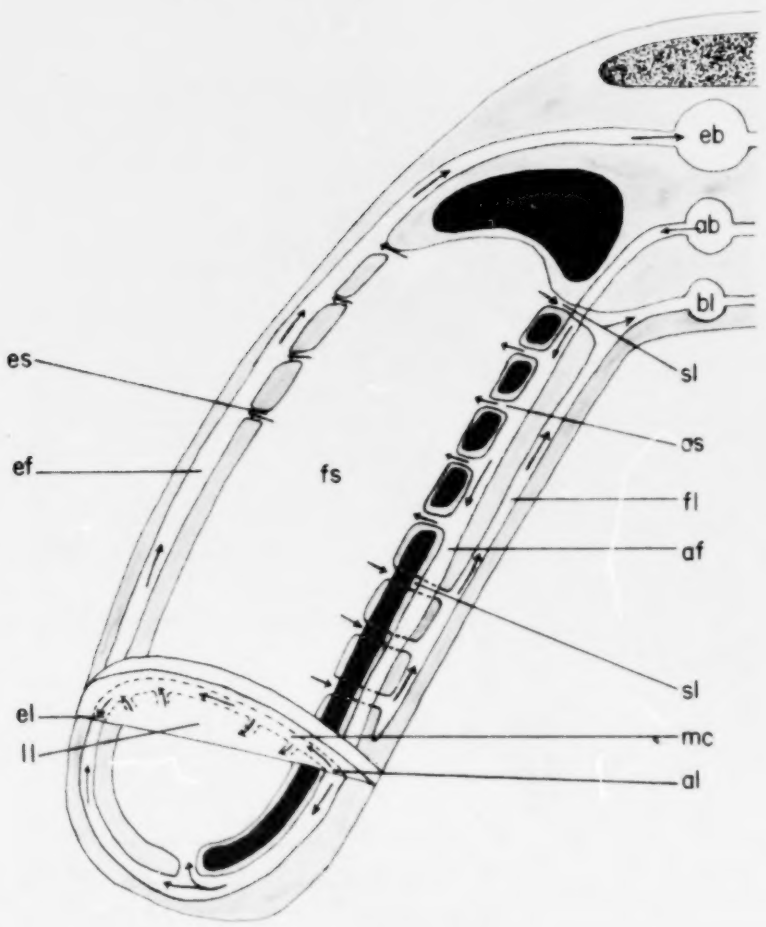

Figure 8. The patterns of fluid flow through the teleost gill with arrows indicating the direction of flow: eb, efferent branchial artery; ab, afferent branchial artery; bl, branchial lymphatic vessel; sl, sinus-lymphatic vessel; as, afferent sinus vessel; fl, filamental lymphatic vessel; af, afferent filamental artery; mc, marginal channel of the lamella; al, afferent-lamellar arteriole; ll, lamellar lacumae; el, efferent lamellar arteriole; ef, efferent filamental artery; es, efferent sinus vessel; fs, filamental sinus.

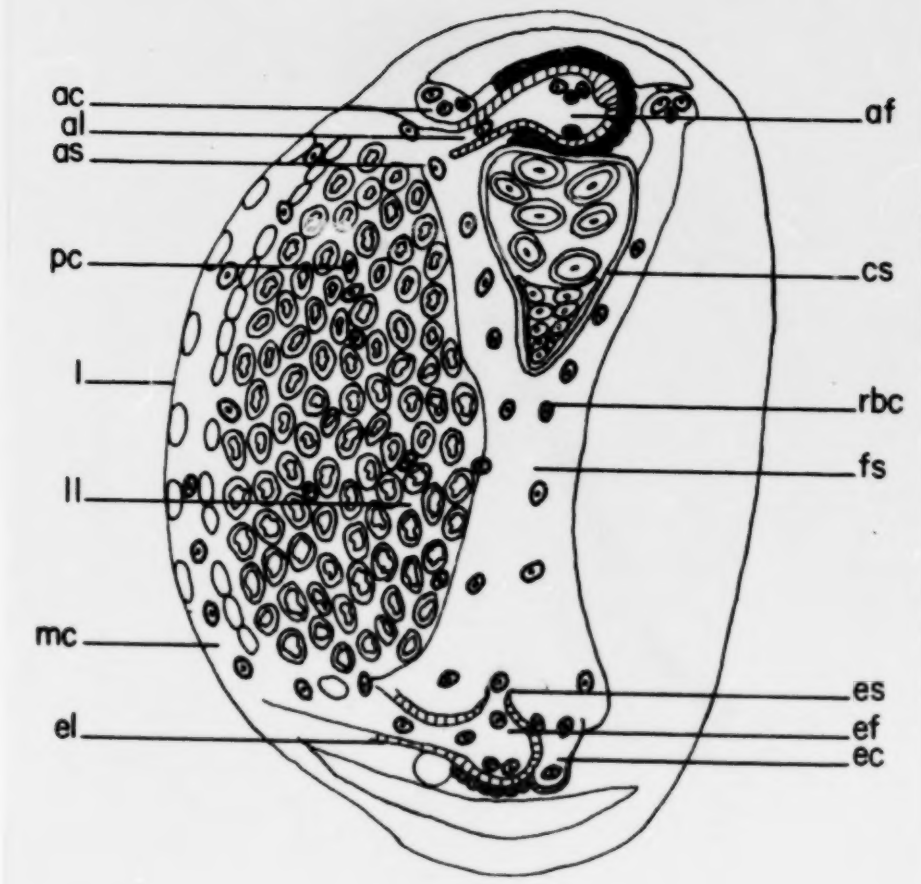

Figure 9. Diagrammatic representation of a cross-section of a gill filament: cs, cartilaginous support; rbc, red blood cell; ec, efferent collateral vessel; l, lamella; pc, pillar cell; ac, afferent collateral. All other abbreviations are as in Figure 8.

(b) filamental sinus, and (c) direct connection between the afferent and efferent filamental arteries at the tip of the filament. When acetylcholine was added to the salt solution bathing a filament, they found that the blood flowed through the filamental sinus and around the tip of the filament. Addition of adrenalin, on the other hand, caused all of the blood to flow through the lamellae. The absence of a normal, unidirectional, afferent-to-efferent pressure gradient in their experiments makes it difficult to determine the true physiological significance of the flow patterns described. In our study, using approximately normal afferent-to-efferent pressure gradients, and with the acetylcholine or epinephrine in the perfusing fluid rather than applied to the outside of the gill, the results we obtained were similar to those of Steen & Kruysse (1964). The effects of epinephrine and acetylcholine, however, were not as absolute as those reported by these investigators. For example, perfusion with India ink containing acetylcholine did not completely eliminate the flow of fluid through the lamellae, and perfusion with epinephrine did not result in 100 per cent lamellar flow. The effect of atropine indicates that there may be some tonic cholinergic regulation of gill blood flow. Since we observed only small differences between the distribution of India ink in control and acetylcholine-treated gills, we believe that the pattern and/or regulation of blood flow through teleost gills is primarily under adrenergic control. In rainbow trout the pattern of blood flow through the gill seems to vary from "purely cholinergic" flow (exclusively through the filamental sinus) to "purely adrenergic" flow (exclusively through the lamellae). The site of control of gill blood flow pattern probably involves some combination of the lamellar arterioles and the sinus vessels. Acetylcholine, which not only causes blood to flow through the filamental sinus but also decreases the flow rate (Ostlund & Fänge, 1962), probably acts solely to cause vasoconstriction of the lamellar arterioles. Epinephrine gives rise to greater blood flow through the high-resistance lamellar circulation and increases lamellar flow rate. Thus epinephrine must cause both vasoconstriction of the sinus vessels and vasodilation of the lamellar arterioles.

Sodium uptake by isolated-perfused gills of rainbow trout: As indicated above, the initial aim of the perfusion studies was to obtain information on the level or rate of sodium transport by gill epithelial cells. In any analysis of the net uptake or excretion of ions by the gill, four major factors must be taken into account. They are: (1) the mechanism by which the ions are moved, e.g., active transport, exchange diffusion, etc., (2) the surface area across which exchange can occur, (3) the duration of exposure between the vascular fluid (or perfusion fluid) and the fluid bathing the gill, and (4) the

concentration gradient against which the ions must move. Experiments designed to examine the net sodium flux across isolated-perfused trout gills with respect to each of these parameters. Some information on the mechanisms involved was obtained by examining the effects of metabolic inhibitors on net flux of sodium. The effective surface area available for exchange was studied by histological examination of gills which had been perfused with India ink containing various vasoactive agents (as noted above), and the rate of fluid flow through the branchial vasculature was used as a measure of the duration of exposure between the perfusion fluid and the water. The concentration gradients for sodium across the gills were determined in all experiments and were kept relatively constant during any single experiment. The perfusion system used was similar to that described above. Gills were isolated and perfused with Ringer solution to clear them of blood. Following this initial perfusion, a typical experiment was conducted as follows. The free end of the outflow tube was raised to a height of approximately 20 cm above the gill in order to establish a normal afferent-to-efferent pressure gradient. The gill was then placed in 50 ml of fresh 1 per cent Ringer solution and a clean 100 µL pipette was placed over the free end of the outflow tube. A stop watch was started and the time required to collect 100 µL of fluid was recorded. Fifty-microliter aliquots of the stock perfusion fluid and the fluid collected after passing through the gill were taken for sodium analyses to provide control data. The valve on the perfusion apparatus was then turned to allow the experimental perfusion fluid to flow through the gill. A 100 µL sample of the fluid flowing from the efferent cannula was collected and discarded. The gill was then placed in 50 ml of fresh 1 per cent Ringer solution, a clean 100 µL pipette was placed over the free end of the outflow tube and the time required for the collection of 100 µL of fluid was determined and recorded. Aliquots of the experimental perfusion fluid and the fluid collected after passing through the gill were again taken for sodium analyses. The sodium concentration of the bath solution was monitored to check for leaks of perfusion fluid from the gills. If the bath concentration increased by more than 1.5 m-equiv. Na^+/L, all data for that experiment were discarded. Sodium was measured using a Coleman Model 21 flame photometer and a Coleman Model 22 Galv-ometer. All dilutions were made with 0.02% Sterox SE in distilled water. Sodium concentration was read as per cent transmission and converted to m-equiv. Na^+/L using a standard curve. As a constant check on the accuracy of the readings, the solutions were read in the following order: bland, 150 m-equiv./L standard, bland, sample, bland, etc. The accuracy with which the sodium concentration of a given sample could be determined was ± 1.0 m-equiv. Na^+/L. When

Table VI

Sodium uptake as affected by variable sodium concentrations, inhibitors, and vasoactive agents. The rates of sodium uptake are given for gills perfused with experimental solutions containing different sodium concentrations, inhibitors, and vasoactive agents.

Solution	Na^+ concentration in perfusion fluid mEq/L ± S.E.	Rate of Na^+ uptake in μEq/min ± S.E.
100% Ringer	156.2 ± 0.5	-0.026 ± 0.014
Choline Ringer 1	139.2 ± 0.4	0.068 ± 0.025
Choline Ringer 2	124.0 ± 0.6	0.142 ± 0.040
Choline Ringer 3	116.1 ± 1.1	0.122 ± 0.040
Choline Ringer 4	83.9 ± 0.0	0.189 ± 0.110
Choline Ringer 4 + 10^{-3}M Atropine	81.4 ± 0.9	0.223 ± 0.070
Choline Ringer + 10^{-5}M Epinephrine	81.2 ± 0.8	1.154 ± 0.182
Choline Ringer 4 + 10^{-4}M Ouabain	86.3 ± 1.3	0.034 ± 0.017
Choline Ringer 4 + 10^{-4}M Cyanide	84.9 ± 1.1	0.086 ± 0.033
Choline Ringer 4 + 10^{-4}M Iodoacetate	83.3 ± 1.2	0.201 ± 0.064
100% Ringer + 10^{-5}M Epinephrine	150.4 ± 1.8	0.388 ± 0.137

isolated trout gills were perfused with 100 per cent Ringer solution, there was a small but significant loss of sodium to the bathing medium; however, in all cases where the perfusion fluid had concentrations of sodium less than that of normal plasma or when epinephrine was added to the perfusion fluid, sodium uptake was significantly greater than zero (Table VI). When the sodium concentration of the perfusion fluid was reduced to 139, 123, 115 and 84 m-equiv. Na^2/L (choline Ringer 1-4) by replacing sodium chloride with choline chloride, it was found that the rate of sodium uptake was inversely related to the sodium concentration of the perfusion fluid (Figure 10). The internal sodium concentration at which no net uptake or loss of sodium would be expected to occur (152 m-equiv./L) is very close to the normal plasma sodium concentration (155.4 ± 1.2 m-equiv./L) which was found in the rainbow trout studied. Addition of atropine (10^{-3}M) to the perfusion fluid did not result in any significant change in the rate of sodium uptake by gills perfused with choline Ringer 4. The most rapid uptake of sodium occurred when gills were perfused with choline Ringer 4 which contained 10^{-5}M epinephrine. The rates of sodium uptake by gills perfused with choline Ringer 4 containing 10^{-4}M ouabain or cyanide were 86 and 65 per cent lower, respectively, than the rates of uptake by gills perfused with choline Ringer 4 which contained no inhibitor. The differences were significant at the P = 0.025 and P less than 0.10 levels, respectively. In a similar experiment it was found that iodoacetate had no significant effect on the rate of sodium transport by isolated gills. The various perfusion solutions used in this study not only affected the rate of sodium uptake by the gills, but also altered the rates and patterns of fluid flow through the gills. These data are summarized in Table VII. When the experimental perfusion solution used was the same as the control solution (100 per cent Ringer), there was a significant increase in flow rate. When epinephrine was added to the choline Ringer 4 and 100 per cent Ringer perfusion fluids, the flow increased by 48 and 177 per cent of the control values, respectively. Flow rates for all of the other perfusions were significantly less than control rates, except that no significant change in flow rate occurred when gills were perfused with choline Ringer 2 or choline Ringer 4 containing atropine.

General discussion and summary of research on uptake of sodium by trout gills: When isolated gills were perfused with Ringer solution which had a sodium concentration equal to or above that found in normal trout plasma, we observed a slight but significant loss of sodium to the bath solution. When the sodium concentration of the perfusion fluid was below that of normal trout plasma, we were able to measure a net transfer of sodium from the bath into the vascular

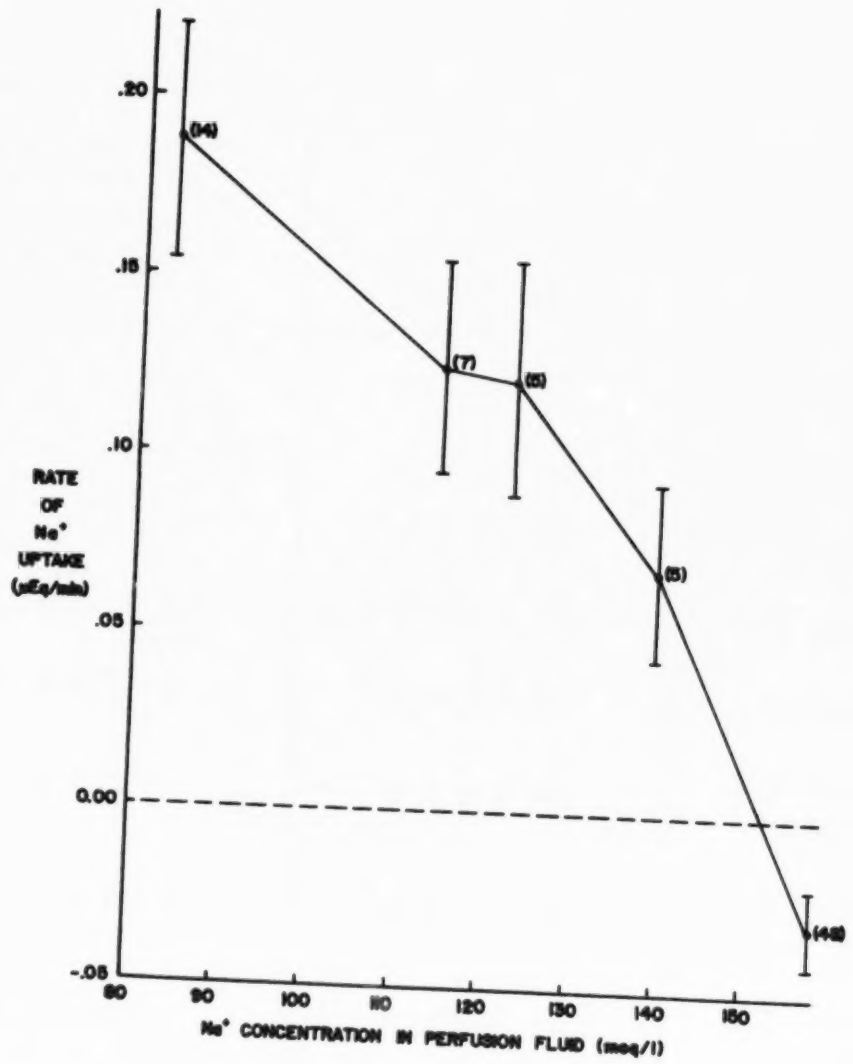

Figure 10. The rate of sodium uptake by gills as a function of the sodium concentration of the perfusion fluid.

Table VII

Factors affecting rate of fluid flow through isolated trout gills. The rates of fluid flow through gills during control (100% Ringer) perfusions are given along with the per cent changes in flow rates during perfusion with experimental solutions containing different sodium concentrations, inhibitors, and vasoactive agents.

Solution	Initial rate of fluid flow μL/min ± S.E. (100% Ringer solution)	Per cent change in flow rate (experimental perfusion)
100% Ringer	24.7 ± 2.6	+ 17.2 ± 2.6
Choline Ringer 1	21.9 ± 2.7	- 39.4 ± 11.9
Choline Ringer 2	17.2 ± 2.5	- 43.2 ± 7.6
Choline Ringer 3	17.8 ± 2.6	- 33.7 ± 10.1
Choline Ringer 4	22.2 ± 2.2	- 65.7 ± 7.3
Choline Ringer 4 + 10^{-3}M Atropine	33.3 ± 3.9	- 1.9 ± 14.0
Choline Ringer 4 + 10^{-5}M Epinephrine	34.5 ± 6.4	+ 48.8 ± 15.2
Choline Ringer 4 + 10^{-4}M Ouabain	15.6 ± 4.4	- 66.4 ± 12.4
Choline Ringer 4 + 10^{-4}M Cyanide	19.5 ± 2.5	- 74.1 ± 4.8
Choline Ringer 4 + 10^{-4}M Iodoacetate	21.6 ± 3.8	- 31.6 ± 15.1
100% Ringer + 10^{-5}M Epinephrine	22.7 ± 3.7	+177.6 ± 40.3

system of the gills. This net inward transfer of sodium was against concentration gradients as high as 100:1, and the rate of uptake varied inversely with the sodium concentration of the perfusion fluid. Thus it appears that the gills themselves are able to regulate the net sodium flux between the vascular system and the fluid bathing the gill and that this autoregulatory action is dependent upon the concentration of sodium in the perfusion fluid. The inhibition of sodium uptake by ouabain and cyanide indicated that sodium uptake by isolated, perfused rainbow trout gills is an ATP-dependent process and that much of the ATP used is derived from oxidative metabolism. The complete lack of inhibition by iodoacetate suggests that glycolysis per se is not required for sodium uptake by the gill. These results, along with those obtained by Kamiya (1967) using salt-water-adapted eels, suggest that glycolysis may be generally unimportant as a metabolic pathway in the gills of fish. The data and observations recorded in Table VIII indicate that the rate of sodium uptake is not primarily dependent upon either flow rate or flow pattern through the gill. Two factors which do appear to affect the rate of net sodium movement across the gill are the sodium concentration of the perfusion fluid and epinephrine.

Deviations of the internal or vascular sodium concentration from normal plasma levels may alter the rate and direction of net sodium movement by either of two ways. Decreased sodium may directly stimulate and increased sodium may directly inhibit the sodium pumping mechanism present in gill epithelial cells. Alternatively, the sodium pump may be continuously active at a level or rate which just balances the normal sodium loss from the fish, thus a decrease in the internal sodium concentration would decrease the passive loss of sodium across the gill and result in a net uptake of sodium. The converse would be true when the internal sodium concentration is above the normal level. The fact that gills perfused with 100 per cent Ringer solution containing 10^{-5}M epinephrine took up sodium at the rate of 0.388 μ-equiv./min as opposed to a net loss of 0.026 μ-equiv./min in the absence of epinephrine indicates that epinephrine has a direct stimulatory effect on the sodium pumping mechanism. This direct action of epinephrine was also evident when the sodium uptake rate by gills perfused with choline Ringer 4 containing epinephrine was compared with that by gills perfused with choline Ringer 4 alone. It is of interest to note that the rate of sodium uptake by gills perfused with choline Ringer 4 containing epinephrine is much greater than the rate of sodium uptake by either gills perfused with 100 per cent Ringer containing epinephrine or by those perfused with choline Ringer 4 alone. The fact that the combined effects of low sodium

Table VIII

Factors affecting pattern of fluid flow through isolated trout gills. This table gives the rates of sodium uptake, the per cent changes in the rates of fluid flow, and the patterns of fluid flow through gills perfused with experimental solutions containing different sodium concentrations, inhibitors, and vasoactive agents.

Perfusion fluid	Rate of sodium uptake (μEquiv./min)	Per cent change in flow rate	Flow pattern
100% Ringer	-0.026	+ 17.2	filamental sinus
Choline Ringer 1	+0.068	- 39.5	filamental sinus
Choline Ringer 2	+0.122	- 43.2	filamental sinus
Choline Ringer 3	+0.125	- 33.7	filamental sinus
Choline Ringer 4	+0.189	- 65.7	filamental sinus
Choline Ringer 4 containing atropine	+0.223	- 1.9	lamellar
Choline Ringer 4 containing epinephrine	+1.154	+ 48.8	lamellar
Choline Ringer 4 containing ouabain	+0.034	- 66.4	filamental sinus
Choline Ringer 4 containing cyanide	+0.086	- 74.1	filamental sinus
Choline Ringer 4 containing iodoacetate	+0.201	- 31.6	filamental sinus
100% Ringer containing epinephrine	+0.388	+177.6	lamellar

concentration and epinephrine on sodium uptake are more than additive has been interpreted to mean that these two stimuli act at different levels on the sodium uptake mechanism. For example, there may be two separate sodium pumping mechanisms, one which transports sodium into the filamental sinus which is most responsive to low internal sodium, and another which transports sodium into the lamellae and is most responsive to epinephrine. The similarity of the sodium uptake rates during perfusions with choline Ringer 4 and choline Ringer 4 containing 10^{-3}M atropine indicates that there is no cholinergic inhibitory mechanism acting to oppose the stimulatory effect of epinephrine. In summary, the experiments reported above strongly indicate that the uptake of sodium by isolated-perfused rainbow trout gills is an oxidative metabolism-dependent, ATP-dependent system which is controlled by the sodium concentration of the perfusion fluid and by epinephrine, and which is generally independent of the rate and pattern of fluid flow through the gill.

Uptake of dieldrin by isolated perfused gills of rainbow trout: During the course of our experiments with isolated-perfused gills, the Fisheries and Wildlife Department at Michigan State University allowed us the use of a gas chromatograph on a limited time basis. We chose to work with the insecticide dieldrin in order to take advantage of the experience and advice offered by Dr. H. Johnson and coworkers in that department. Dieldrin, a cyclodiene, is a widely used pesticide with a relatively long residual life in the environment. Aquatic organisms such as fish may assimilate dieldrin from water through the gill membranes or skin or via the alimentary tract. Holden (1962, 1965), Premdas and Anderson (1963), Crosby and Tucker (1966), and Lenon (1968) have all suggested that uptake of DDT and related insecticides including dieldrin occurs primarily by way of the gills. No specific data on gill uptake were presented by these workers, but the extremely rapid uptake and dispersal of DDT in fish led them to conclude that the gills are the chief port of entry and the blood is the chief means of transport of assimilated insecticides. The purpose of our experiments was to investigate the transfer of dieldrin from the environmental water into the vascular system of isolated-perfused gills of rainbow trout.

Gill arches were surgically removed and attached to a gill perfusion apparatus as described above. The arches were perfused initially with Ringer solution containing glucose for about 15 mins to clear the filaments of blood cells, and during this time the gills were bathed with the nutrient Ringer solution. Dieldrin was dissolved in ethanol (10 mg/ml) and bath solutions containing approximately 1000 µg/L

were prepared by adding 0.1 ml of dieldrin stock solution to 1 liter of dechlorinated tap water. Bath solutions were prepared at least 24 hours prior to use. About 80 ml were then put into a glass beaker and stirred continuously with a teflon-coated stirring bar. Analyses of samples indicated that during the 9-min equilibration period a variable amount of dieldrin was volatilized or adsorbed on the beaker walls so that the initial concentrations to which the gill arches were exposed varied from one experiment to another. All experiments were performed in a walk-in refrigerator at 13°C.

To closely mimic normal physiological conditions the initial perfusion fluid used was heparinized plasma. Since it was difficult to obtain sufficient volumes of plasma from a single fish for perfusion and we disliked the idea of using pooled samples from several donor fish, a fish Ringer solution containing glucose was substituted as the perfusion fluid. When no dieldrin appeared in the perfusate of Ringer-perfused gills, we used TC-199 instead of the Ringer solution. The synthetic tissue culture medium (TC-199) is a product of Difco Co., Detroit, Michigan, which contains salts, vitamins and several amino acids but no protein.

In a typical experiment a 1 ml aliquot of the dieldrin bath was taken for analysis at the time the gills were placed in the solution, and a second aliquot was taken after 1 ml of perfusate had been collected from the gill. A 1 ml aliquot of the perfusion fluid was also taken for analysis. For determinations of the amount of dieldrin adsorbed on or bound to gill tissues, gills were removed from the bath, flushed with Ringer solution for 3-5 min, weighed, and then digested with 20 per cent alcoholic KOH for 1 hr at 76°C. For analytical consistency all solutions analyzed were subjected to the same digestion procedure as the gill tissues even though not all of the samples contained protein material. After digestion all samples were individually extracted with petroleum ether from which water was removed using anhydrous sodium sulfate. The dieldrin content of the final solution was determined by gas chromatography. In our initial analyses ether extracts were run through a Florisil column to eliminate co-extractives, and many of the smaller peaks in the chromatogram were eliminated. This procedure was deemed unnecessary and was discarded in the studies reported here. The chromatograph used was an Aerograph HY-FL model 600-C (Wilkens Inst. & Res. Inc.) with a proportional isothermal temperature control (Model 328) and an electron capture detector. The 5' × $\frac{1}{8}$" pyrex glass column was packed with 3 per cent QF-1 on 100/120 mesh Gas-Chrom Q. Column temperature was 180°C and gas (nitrogen) flow 40 ml/min.

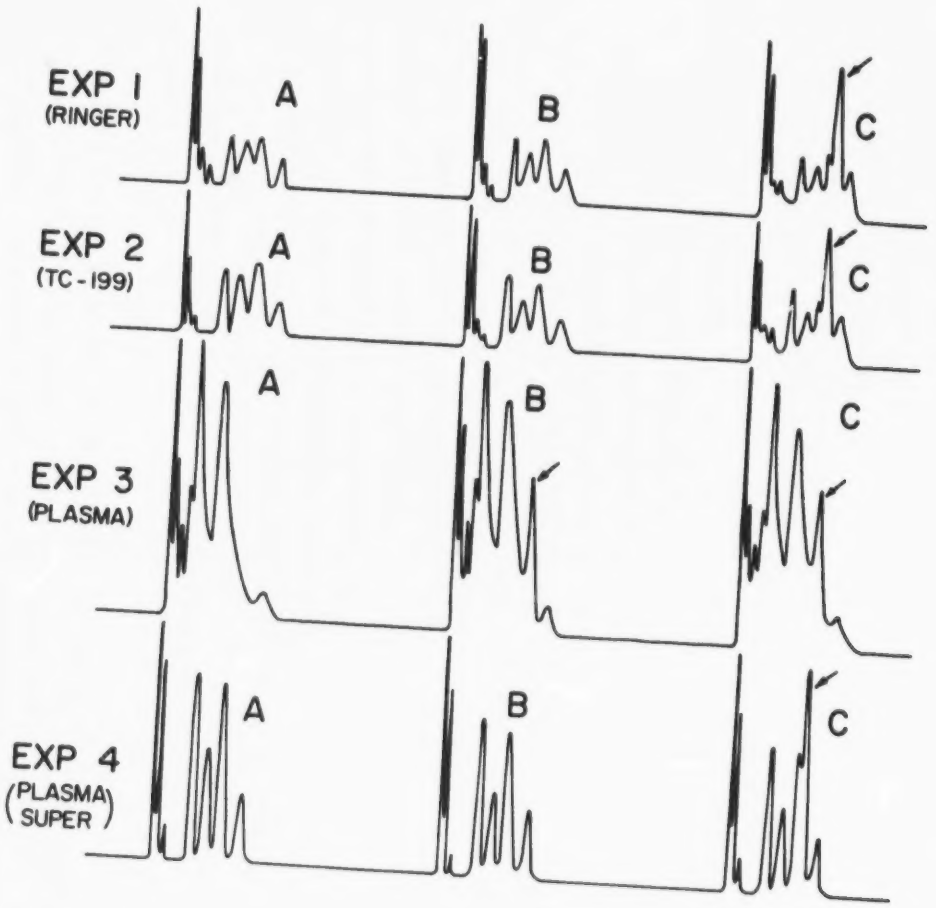

Figure 11. Gas chromatography tracings of (A) perfusion fluid, (B) perfusate, and (C) perfusate to which a known amount of dieldrin was added (spiked), excepting that experiment 3C is spiked perfusion fluid. In experiments 1, 2 and 3 the gills were perfused with Ringer solution, TC-199, and rainbow trout plasma respectively. Tracings for experiment 4A and B are of the supernatant solutions which remained after plasma samples had been treated with barium hydroxide-zinc sulfate and centrifuged to remove protein. Experiment 4C is a chromatogram for spiked supernatant solution. Dieldrin peaks are indicated with solid black arrows.

Results: Typical gas chromatography tracings of the solutions analyzed in the experiments (Figure 11) show that no change occurred either in Ringer solution (experiment 1A, B) or in TC-199 (experiment 2A, B) during passage through isolated gills. That none of the peaks which appear in the tracings of the experimental solutions represented dieldrin is shown by tracings of spiked samples (experiment 1C and 2C). When isolated gills perfused with plasma were placed in a bath of tap water containing dieldrin, the insecticide was found in the plasma perfusate (experiment 3B). Tracings of plasma perfusate from gills which were not exposed to dieldrin were identical to those for non-perfused plasma (experiment 3A), whereas samples of spiked non-perfused plasma (experiment 3C) were identical to those for plasma from dieldrin-exposed gills (experiment 3B). Although the tracings are not shown, spiking of plasma from dieldrin-exposed gills caused an increase in size or height of the dieldrin peak and confirmed the original presence of dieldrin in these samples.

The proteins in two samples of plasma perfusate from gills exposed to dieldrin and in a sample of nonperfused plasma were preciptated using barium hydroxide and zinc sulfate (Somogyi procedure). Gas chromatographic tracings of the supernatant fluid from these samples (Figure 11, experiment 4A, B) indicate that the dieldrin present in the plasma samples was preciptated along with the plasma proteins. Large amounts of dieldrin were found adsorbed on or bound to perfused gills (Table IX) with relatively greater quantities being found associated with plasma-perfused gills than those perfused with Ringer solution. The dieldrin concentration in the plasma perfusate was uniformly less than that in the solution which bathed the gills; and over the range of concentrations tested, the amount of dieldrin transferred across isolated gills appeared to be dependent upon the concentration of the insecticide in the bath.

Discussion and summary: We have clearly demonstrated that dieldrin can be transferred from environmental water into the vascular system of isolated perfused gills of rainbow trout. This transfer occurred only when protein, or more probably lipoprotein, was present in the perfusion fluid. Since the dieldrin concentration in the plasma perfusate from gills was always less than that of the bath fluid, a gradient for inward diffusion existed and there is no need to postulate any other mechanism for inward movement of the insecticide by the gill. The greater quantities of dieldrin found in gills perfused with plasma than in those perfused with Ringer solution may indicate either a greater entry of dieldrin into epithelial cells of gills perfused with plasma and a consequent greater internal binding of the insecticide or inadequate

flushing of the gill lamellae prior to analyses. As the plasma used had been obtained by bleeding the fish from which the gills were taken, it is probable that epinephrine was released into the blood during the blood letting procedure. Epinephrine causes high blood flow into lamellae of perfused gills and it may be that the lamellae were not flushed out by the Ringer solution, which contained no epinephrine.

Table IX

Summary of information on amount of dieldrin bound to perfused gills and effect of concentration of dieldrin in bath on dieldrin transfer by gills. Concentration of dieldrin is given as parts per billion (μg/L).

Perfusion fluid	Number of perfusions	Concentration of dieldrin in bath	Dieldrin bound to gill	Concentration of dieldrin in perfusate
Ringer	2	none	no	none
Ringer	8	119-339	yes[a]	none
TC-199	2	none	---[b]	none
TC-199	2	289	---[b]	none
Plasma	2	none	no	none
Plasma	9	169-515	yes	64-220

[a]But much less than that found in plasma perfused gills.

[b]Not analyzed for dieldrin.

Lipoprotein is present in mammalian blood, and evidence has been accumulating that much of the lipid material in human plasma is combined with protein. This may also be true for fish plasma. We suggest that dieldrin and other related insecticides diffuse through

gills of fishes and are dissolved in the lipid portion of plasma lipoprotein, in which form they are transported to and become dissolved primarily in the lipid portion of the various tissues. Since the insecticide is much more soluble in lipid than in water, the tissue concentrations can attain levels far above that of the environmental water independent of any active transport mechanism. Little is known about the pathways used for the excretion of dieldrin, but since release is rapid (Gakstatter and Weiss, 1967; Lenon, 1968) it appears unlikely that excretion of most of dieldrin is dependent upon lipid turnover in the tissues.

Effects of some insecticides and MS-222 on isolated-perfused gills of trout: Gills of rainbow trout were prepared for perfusion as previously described. The gills were initially bathed with 1 per cent Ringer solution and perfused with 100 per cent Ringer to clear them of blood. Gills were then placed in a fresh 1 per cent Ringer bath and perfused with Ringer solution in which choline chloride was substituted for NaCl so that the solution contained only 80 per cent of the normal amount of sodium (control). After control flow rates were determined, they were placed in a fresh 1 per cent Ringer bath which contained the test chemical and perfused with the low sodium Ringer solution (experimental). To determine the pattern of gill blood flow under the different experimental conditions, gills were perfused with an India ink solution and then prepared for histological examination as described above. No gills exposed to malathion were perfused with India ink.

Table X

Acute effect of various chemicals on rate of fluid flow through isolated-perfused gills of rainbow trout. Flow rates are in μL/min for the control and experimental periods as described in the text.

Conditions	n	Control	Experimental	Per cent change
Untreated gills	11	37	34	10
Dieldrin (1 ppm)	10	34	27	21
Methoxychlor (1 ppm)	6	33	30	8
Rotenone (1 ppm)	6	41	25	39
MS-222 (100 ppm)	10	38	28	24
Malathion (5 ppm)	6	35	28	20

Date for mean flow rates in μL/min during the control and experimental periods are presented in Table X. In each case the mean flow rate was lower during the experimental than during the preceding control period. All differences excepting those for untreated gills and those exposed to methoxychlor were statistically significant ($p < 0.10$).

With respect to patterns of blood flow through gills, histological sections of gill filaments were examined and the number of lamellae which contained ink were noted. For example, some 2348 sections of lamellae from one gill were observed, and 1865 contained ink; thus, 79 per cent of the lamellae for that particular gill exhibited lamellar flow. The other data presented in Table XI were obtained in the same manner.

Table XI

Acute effect of various chemicals on pattern of blood flow through isolated-perfused gills of rainbow trout. Data represents per cent of lamellae perfused with India ink and values were obtained as described in the text.

Control	Rotenone	Methoxychlor	Dieldrin	MS-222
79	92	61	94	83
70	94	81	97	90
92	82	85	93	98
81	82	95		
	92			
	98			
$\overline{X}$				
80	90	80	94	90

Statistics: Rotenone > Control, $p = 0.057$; Dieldrin > Control, $p = 0.028$; Methoxychlor = Control, $p > 0.343$; MS-222 = Control, $p = 0.114$.

All chemical compounds tested except methoxychlor had an acute effect on isolated gills and reduced the flow of perfusion fluid through them. In this same series of experiments we measured the sodium content (flame photometry) of the afferent and efferent perfusion fluids and, using appropriate calculations, we were able to detect changes in inward sodium transport per unit of time. In many cases the differences in the concentration of sodium in the afferent and efferent solutions were so small as to be within the range of accuracy of the analytical procedure employed. To determine the effect of the test chemicals on sodium transport by isolated gills, it will be necessary to use radiosodium, and we plan to carry out experiments of this nature in our laboratory.

It should be emphasized that the decreases in flow rates occurred at constant perfusion pressure, which means that resistance to fluid flow through gills was increased. Blood flow from afferent to efferent filamental vessels in fish gills is via the filamental sinus, apical and lamellar lacunae pathways. It has been assumed that the presence of pillar cells in lamellar lacunae make this a high resistance pathway for fluid flow. Smooth muscle, capable of controlling blood flow patterns, has been found in the walls of the afferent and efferent vessels of both the filamental sinus and the secondary lamellae.

Although increased lamellar perfusion and decreased flow rates generally go hand-in-hand, the addition of epinephrine to the perfusion fluid was shown to give rise to both an increase in lamellar perfusion and an increase in the overall flow rate through the gill, as noted above. Thus the substances tested may have exerted their effects at one or more of the various control sites in the afferent and efferent branchial vasculature.

The decreased flow of fluid through gills treated with rotenone and dieldrin appears to result from a shift of flow into the high resistance lamellar route. Flow rates for methoxychlor treated gills were not significantly different from those which occurred in controls and both groups had similar percentages of lamellar perfusion. The absence of a significant change in the pattern of ink flow through MS-222 treated gills may have been due to a change in the resistance of the other blood flow pathways, or the data may represent an artifact due to the small number of observations and the variability of the gills examined.

In summary: Isolated gills of rainbow trout were perfused with a sodium deficient Ringer solution in the presence of various pesticides and MS-222 and flow rates were determined. This was followed by

perfusion with India ink and preparation for histological determination of fluid flow patterns through the branchial vasculature. It was found that short-term (acute) exposure to dieldrin, rotenone, malathion and MS-222 resulted in a statistically significant reduction in perfusion flow rate through the isolated gills. Exposure of gills to 1 mg/L methoxychlor was without effect. Results of India ink perfusions indicated that decrease in rate of fluid flow through the gills correlated well with increased lamellar perfusion.

SECTION V

ACKNOWLEDGMENTS

This research was carried out in the Comparative Physiology Laboratory, Department of Physiology, Michigan State University, East Lansing, Michigan 48823.

The research was supported in part by the Michigan Agricultural Experiment Station. The rainbow trout used in the experiments were obtained from the Michigan Department of Natural Resources through the cooperation of Dr. L. N. Allison, Fish Pathologist, Grayling Research Station, Grayling, Michigan.

The gas chromatograph used in experiments with dieldrin was made available through the courtesy of Dr. Howard Johnson, Department of Fisheries and Wildlife, Michigan State University.

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SECTION VIII

PERSONNEL

Staffing: All persons, other than the Principal Investigator and R. C. Hunter, who were formerly or are now employed on this grant project have been graduate students in the Department of Physiology at Michigan State University. Appointments were usually on a one-half time basis and were entitled "Special Graduate Research Assistant." Appointees, along with a brief biographical sketch, are listed in chronological order of employment. Dates of employment are in parentheses.

Janet Gillette, B.S. Biology, Bridgewater State College; M.S. Physiology, Michigan State University, 1967. Currently employed as an instructor in Biological Science at University of Pittsburgh at Greensburg, Pennsylvania, and is working toward Ph.D. at University of Pittsburgh. (6-66 to 9-67)

C. Hill, B.S. Chemistry-Physics, Wisconsin State College, Superior; B.S. and M.S. Fish and Wildlife Management, Montana State University; Ph.D. Physiology, Michigan State University, 1967. He is currently employed as Assistant Professor, Department of Biology, California State College, Long Beach, California. (6-66 to 7-67)

Mack Holt, B.S. Biology, Ft. Valley State College; withdrew from graduate program in Physiology at Michigan State University, 2-15-68. Current address is unknown. (9-67 to 2-68)

Wayne Price, B.S. Biological Sciences, Michigan State University; withdrew from Master's program in Physiology at Michigan State University, December 1968. Currently in U.S. Armed Forces. (3-68 to 12-68)

B. D. Richards, B.S. Zoology, University of Michigan; M.S. Biology, Florida State University; Ph.D. Physiology, Michigan State University, 1969. Currently employed as Assistant Professor, Department of Biological Sciences, Illinois State University, Normal, Ill. (9-67 to 6-68)

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R. C. Hunter, B.S. Physiology, Michigan State University; M.B.A. Michigan State University, 1969. Currently employed by Pfizer Drug Company, New York, New York. Mr. Hunter was a U.S. Navy veteran, a former corpsman, and was hired on an hourly basis as a technician. (6-69 to 12-69)

Kenneth Olson, B.S. Biochemistry, University of Wisconsin at LaCrosse; M.S. Physiology, Michigan State University, 1970. Currently a graduate student in Physiology at Michigan State University. (6-69 to present)

R. L. Walker, B.S. Biological Sciences, Alma College. Currently a graduate student in Physiology at Michigan State University. (6-70 to present)

1 Accession Number	2 Subject Field & Group	SELECTED WATER RESOURCES ABSTRACTS INPUT TRANSACTION FORM

5 Organization

Michigan State University, Department of Physiology, E. Lansing, Mi. 48823

6 Title

Toxic action of water soluble pollutants on freshwater fish

10 Author(s)	11 Date	12 Pages	15 Contract Number
Dr. Paul O. Fromm	12-5-70	56	
	16 Project Number: 18050DST		21 Note

22 Citation

23 Descriptors (Starred First)

Fish*, stress*, chromium, insecticides, ammonia*, sodium transport gill blood flow, nitrogen, fish physiology, nitrogen excretion*

25 Identifiers (Starred First)

Fish*, stress, nitrogen excretion*, fish physiology

27 Abstract

Over a five year period experiments on rainbow trout indicated that exposure to chromium ant to forced exercise caused a transient increase in plasma cortisol. Exposure to ammonia (a) caused a decrease in the rate of total nitrogen excretion and in ammonia excretion (b) caused some histopathological changes in trout gills but oxygen transport by hemoglobin was unaffected (c) caused a very slight increase in urea excretion by trout but a very significant rise in goldfish. Hyperexcitability observed in ammonia-exposed trout was not noticeable in the more resistant goldfish. Ammonia may kill fish by prevention of excretion of normal amounts of endogenous ammonia. Experiments with isolated-perfused gills of trout have shown among other things that (a) gill blood flow patterns are significantly affected by epinephrine (b) when perfused with Ringer solution there was a small but significant loss of sodium into the bath solution, whereas perfusion with sodium-poor Ringer solutions always resulted in a net uptake of sodium (c) perfusion fluid sodium and epinephrine appear to control sodium uptake by the gill (d) transfer of dieldrin into the vascular system occurred only when plasma protein, or more probably plasma lipoprotein was present in the perfusion fluid (e) short term exposure to dieldrin, rotenone, malathion and MS-222 reduced perfusion flow rate through isolated gills but exposure to 1 mg/L methoxychlor was without effect. Decrease in flow rate correlated well with lamellar perfusion.

Abstractor	Paul O. Fromm
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WR:102 (REV. OCT. 1968)
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